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Molecular detection of Dientamoeba fragilis among patients at Raparin Teaching Hospital for Children in Erbil City, Iraq using nested PCR

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ABSTRACT

Dientamoeba fragilis is a globally distributed protozoan parasite that is recognized as a causative agent of gastrointestinal symptoms, particularly within pediatric populations. The incidence of infections has increased in recent years, largely due to the implementation of more sensitive molecular techniques that have enhanced both the detection and diagnosis of this organism. This study aimed to determine the prevalence of *Dientamoeba fragilis* among pediatric patients using nested PCR technique. It represents the first molecular investigation of this parasite in Iraq. A total of 100 stool specimens were collected from children aged between (>1 - 12) years. The analysis revealed higher prevalence rate of 19% using nested PCR compared to 17% observed through primary PCR. The highest infection rate was recorded in the age group of 1 to 3 years. These findings underscore the critical need of enhancing public and clinical awareness regarding *D. fragilis* infections within this country

Keywords: *Dientamoeba fragilis, Prevalence, Pediatric Children, Nested PCR*

1. INTRODUCTION

Dientamoeba fragilis is a flagellated trichomonad protozoan parasite that lives in humans' gastrointestinal tracts and has a global distribution (Windsor and Johnson, 1999, Barratt et al., 2011, Stark et al., 2016). Originally misclassified as an ameba, ultrastructural and phylogenetic studies based on 16S-like rDNA have redefined it as a trichomonad (Camp et al., 1974, Silberman et al., 1996, Munasinghe et al., 2013).

Over the past two decades, *D. fragilis* has emerged as an important enteric pathogen, especially in children (Norberg et al., 2003, Banik et al., 2011, van Kalleveen et al., 2020, Shasha et al., 2024) with reported symptomatic infection rates ranging from 0.3% to 91% diverse populations depending on the region, population, and diagnostic method used in the studies (Barratt et al., 2011, Stark et al., 2016). It is now recognized as being more prevalent than *Giardia intestinalis* and *Entamoeba histolytica* (Crotti et al., 2005, Röser et al., 2013, Pietilä et al., 2019, Shasha et al., 2024).

The parasite has been associated with a variety of gastrointestinal symptoms, including diarrhoea, abdominal pain, flatulence, and anorexia, resembling irritable bowel syndrome (IBS) and often in association with eosinophilia (Grendon et al., 1995, Johnson et al., 2004, Stark et al., 2007). However, its clinical significance remains debated due to frequent detection in asymptomatic individuals, particularly children and adults, making it difficult to establish a clear correlation between infection and clinical symptoms (Jokelainen et al., 2017, Brands et al., 2019, Bamini et al., 2024). Numerous studies have demonstrated that treatment aimed at eradicating the parasite often results in clinical improvement, particularly in symptomatic patients, underscoring the importance of accurate diagnosis and targeted therapy (ter Schure et al., 2013, Stark et al., 2016, van Kalleveen et al., 2020, Pietilä et al., 2023). Despite this, the parasite is frequently overlooked in clinical practice, partly due to diagnostic challenges and the lack of standardized treatment protocols (van Kalleveen et al., 2020).

Diagnostic limitations have hindered accurate prevalence assessment. Traditional methods, such as microscopy of permanently stained smears or cultivation, are labour-intensive and prone to false negatives due to the parasite's fragility and intermittent shedding (Stark et al., 2014, Cacciò, 2018, Abou-Gamra et al., 2024). In contrast, the introduction molecular techniques, particularly those base on PCR, has resulted in a high sensitivity and specificity alternative for detecting *D. fragilis* in patient samples. This advancement has significantly improved our understanding of its epidemiology and determination of its accurate prevalence. These methods are also valuable for studying the parasite's genetic diversity based

on ribosomal DNA (rDNA), which may correlate with pathogenicity (Stark et al., 2011, Cacciò, 2018, Gough et al., 2019). These advances have revealed higher infection rates than previously recognised, especially in pediatric populations (Verweij and Stensvold, 2014).

Transmission of *D. fragilis* is believed to occur primarily via the faecal-oral route, with cyst stages identified in both rodent models and human clinical samples (Munasinghe et al., 2013, Stark et al., 2014, Hall et al., 2024). This transmission mode aligns with its prevalence in high-contact settings like daycare centres, where enteric pathogens spread rapidly among children (Jokelainen et al., 2017, Oliveira-Arbex et al., 2021).

This study was conducted to document the prevalence of *Dientamoeba fragilis* in stool samples collected from pediatric patients attending Raparin Teaching Hospital for Children in Erbil City, Iraq using nested PCR.

2. MATERIALS AND METHODS

STOOL SPECIMENS

One hundred fresh faecal samples were submitted to the parasitology laboratory at Raparin Teaching Hospital for Children in Erbil City, Iraq from attendees which their ages were ranging between one month to 12 years old for both genders from March to August 2023. All fresh specimens without adding fixative were kept in a freezer at (-20 °C) to avoid DNA degradation.

DNA EXTRACTION

DNA was extracted from faecal specimens using the QIAampe DNA stool mini kit (Qiagen, Germany) according to the manufacturer's recommendations.

RCR and DNA SEQUENCING

All (100) collected faecal specimens were submitted to both primary PCR and nested PCR. The parasite's small-subunit ribosomal DNA (SSU rDNA) was applied. For primary PCR, oligonucleotide primers previously described for the amplification of *D. fragilis* SSU rDNA were used: DF400 (5`-TATCGGAGGTGGTAATGACC-3`) and DF1250 (5`-CATCTTCCTCCTGCTTAGACG-3`) (Stark et al., 2005) with expected 887 bp amplicon size. PCR amplification (25 µl) was performed using Master mix (Ampliqon PCR Enzymes & Reagents, Denmark), which included Master mix (12.5 µl); 0.2 µM of each primer; DNA (3 µl); and ddH_2O (6.5 µl), and with a PCRmax Alpha thermal cycler (UK). The following thermocycling profile: 5 min at 95 °C; 40 cycles of 1 min at 95 °C, 1.5 min at 58 °C, 2 min at 72 °C, then a final cycle of 5 min at 72 °C. The nested PCR was performed on 2 µl of the primary PCR product using primers previously designed: DFF2: (5'

CGGGGATAGATCTATTTCATGGC-3') and DFR2: (5'-CCAACGGCCATGCACCACC-3') (Sarafraz et al., 2013) with expected 403 bp

amplicon size. The following thermocycling profile: 5 min at 95 °C; 30 cycles of 30 sec at 95 °C, 30 sec at 60 °C, 30 sec at 72 °C, then a final cycle of 5 min at 72 °C. The PCR product was analysed by electrophoresis on 1.5% agarose. PCR product was purified utilizing Gel purification kit (QIAquick Gel Extraction, Qiagen, Germany) in accordance with the manufacturer's instructions.

The PCR product was sequenced in both directions on an ABI 3730XL nucleotide sequence analyser provided by Macrogen Inc. (Korea). The sequence obtained was edited and aligned using ClustalW algorithm, available through

MUSCLE program within EMBL-EBI

(https://www.ebi.ac.uk/jdispatcher/msa/muscle). The aligned sequences were then compared to those available in the GenBank databases using the (Blastn) tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi), run on the NCBI server.

STATISTICAL ANALYSIS

Statistical analyses were conducted utilising GraphPad Prism software version 9.0.1, with a p-value threshold of less than 0.05 considered statistically significant.

3. RESULTS

A total of one hundred stool samples (56% male and 44% female) were collected from children and tested for *D. fragilis* parasitic infection using both primary and nested PCR techniques. Seventeen stool samples (12% males and 5% females) were identified as positive for the parasite. Additionally, 83 samples returned negative results when assessed using primary PCR methodology. In the context of nested PCR analysis, nineteen stool samples were positive (14% males and 5% females), while 81 samples exhibited negative results. No significant differences observed in the prevalence of infection rates between genders using both PCR techniques with p-values of 0.1262 for primary PCR and 0.8542 for nested PCR. The median age of infected patients was 2.3 years (range >1 – 10 years) and the peak incidence observed among individuals age between 1

and 3 years (**Table 1 and Figure 1**). Additionally, primary PCR did not reveal significant differences among age groups (p=0.0639). In contrast, the nested PCR method indicated a significant difference (p=0.0313). The relationship of infection rates between age groups and gender were unsignificant in both methods with p-values of 0.3854 for primary PCR and 0.2913 for nested PCR.

The ribosomal DNA of the parasite was subjected to sequencing analysis. The resultant sequence data for *D. fragilis* has been submitted to the GenBank database, where it is assigned the accession number PV686485. This sequence exhibits a 100% similarity to the reference sequence associated with accession number OP375684, which is also accessible within the GenBank database.

Table 1. Number of positive specimens according to age groups and gender

Age groups (Years)	+ve Primary PCR %			+ve Nested PCR %		
	Total	Male	Female	Total	Male	Female
< 1	3	3	0	4	4	0
1 - 3	8	6	2	9	7	2
4 - 6	2	1	1	2	1	1
7 - 9	3	2	1	3	2	1
10 - 12	1	0	1	1	0	1

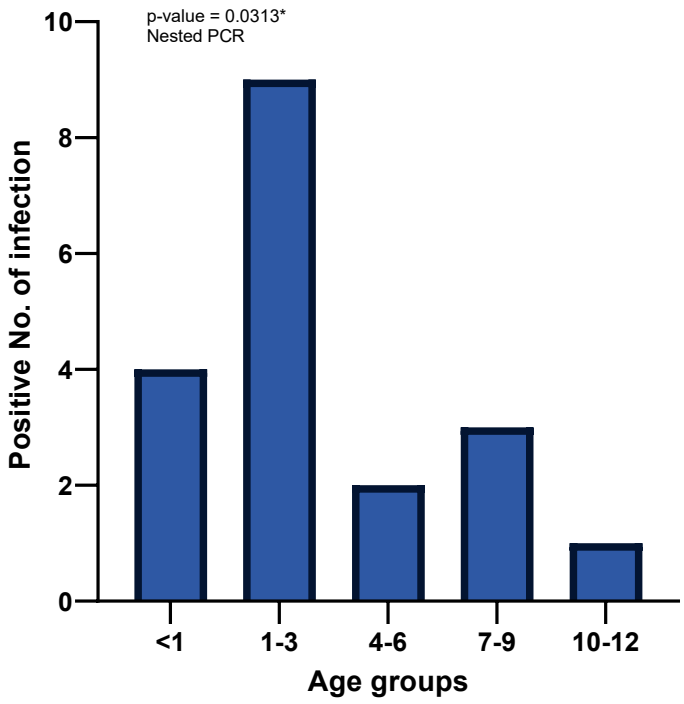

Fig. 1 Differences in positive infections among age groups using nested PCR.

4. DISCUSSION

The current study provides the first documentation of *Dientamoeba fragilis* in stool specimens in Iraq utilising PCR-based diagnostic techniques. All previous researches on this parasite in this country were relied solely on microscopic examinations (Al-Najar, 2006, Guirges, 2006). Investigations into this parasite in Iraq, as well as in numerous other countries, are frequently conducted alongside with other enteric parasites, including *Giardia intestinalis*, *Entamoeba histolytica*, *Cryptosporidium* spp., and *Blastocystis* spp. due to the resemblance of gastrointestinal symptoms, co-infections often common, compounded by a lack of awareness and historical neglect regarding this parasite as a pathogen (Stark et al., 2016,

van Kalleveen et al., 2020).

Using primary and nested PCR, *D. fragilis* was detected in 17% and 19% of specimens, respectively confirming the presence of additional positive samples that primary PCR missed. The detection rates of this parasite using primary and nested PCR techniques highlight the effectiveness of molecular methods in identifying this protozoan parasite over microscopy and culture methods (Ghazanchaei et al., 2012, Sarafraz et al., 2013, Abou-Gamra et al., 2024). Nested PCR assays have proven to be more effective than conventional PCR techniques, especially for amplifying large-sized DNA products. Furthermore, the use of nested PCR increases the sensitivity to detect samples with low copy number DNA in comparison to any single round PCR (Zhang et al., 2023).

Prevalence of this parasite in children was 23.4% attending Children Welfare Teaching Hospital for Pediatrics in Baghdad City, Iraq using microscopy method (Al-Najar, 2006). Frequency was recoded as 2.4% in Iran (Sarafraz et al., 2013), 16% in Tukey (Malatyali et al., 2024), 4% in Egypt (Abou-Gamra et al., 2024) by using nested PCR.

No significant differences in infection rates were observed between genders, which is consistent with the results reported by Oliveira-Arbex et al. (2021). Although studies reported the infection with *Dientamoeba* was higher in female children (Grendon et al., 1995, Crotti and D'Annibale, 2007), however others found the incidence was higher in male children (Norberg et al., 2003, Júlio et al., 2015).

The highest frequency was observed in the 1 to 3 year age group, with statistically significant differences detected through nested PCR analysis. This finding aligns with those reported by Al-Najar (2006) and Jokelainen et al. (2017). Similarly, other studies have identified the highest prevalence of the parasite among children aged 0 to 10 years (Norberg et al., 2003, Stark et al., 2010). In contrast, ter Schure et al. (2013) reported a peak incidence in slightly older children, specifically those aged 4 to 9 years.

Numerous studies have reported various trends in the age-related distribution of *D. fragilis*, influenced by differences in the population studied and the diagnostic methods employed. Some reports suggest that *D. fragilis* is more prevalence in adults and adolescents than in children using macroscopy method (Crotti and D'Annibale, 2007, Stensvold et al., 2007). While, other studies show that prevalence of this parasite is highest in children and adolescents compared to adults using real-time PCR method. For instance, a case-control study in the Netherlands found that the prevalence of *D. fragilis* was significantly highest in individuals under 20 years of age and lower in oldest age group (de Boer et al., 2020). Similarly, a study in Danish day-care centres reported a prevalence of 68.3% in children aged 0-6 years (Jokelainen et al., 2017). This shows that molecular based diagnosis (PCR) could led to higher identification rates of this parasite compared to traditional microscopy method (Röser et al., 2013, Cacciò, 2018).

This parasite exhibits a range in clinical manifestations and has been reported in asymptomatic individuals (Grendon et al., 1995, Windsor et al., 2006). Consequently, genotyping of *D. fragilis* has become an important issue that can link the parasite's pathogenicity to its genetic variation, which may have significant implications for understanding its epidemiology. Phenotyping diversity was observed in growth rate within different isolates of *D. fragilis* cultures collected from patient with gastrointestinal symptoms. The existence of this variation likely to have genetic basis (Barratt et al., 2010). Two *D. fragilis* genotypes (1 and 2) have been identified based on variations in SUU rDNA sequences, with a significant prevalence of genotype 1 in humans and a few animal hosts, but with limited clinical implications (Johnson and Clark, 2000, Johnson et al., 2004, Stark et al., 2005, Windsor et al., 2006, Cacciò et al., 2012, 2016). Although various molecular techniques, including **PCR-RFLP** applied to SSU rDNA (Johnson and Clark, 2000, Peek et al., 2004), **ITS** (Windsor et al., 2006), **C-profiling** within ITS1 and ITS2 region of ribosomal DNA (Bart et al., 2008) and housekeeping genes **actin** and **elongation factor 1 alpha (EF-1α)** (Stensvold et al., 2013) have been utilised to detect genetic variations among different isolates collected in various geographic locations as well as from both symptomatic and asymptomatic cases. Nonetheless, these investigations revealed a restricted degree of polymorphism. Current research has not clearly established a correlation between genetic variation and pathogenicity. The efficacy of genotyping methods in discerning between virulent and avirulent strains of *D. fragilis* remains uncertain (Stark et al., 2016, Cacciò, 2018). Consequently, it is essential to explore additional molecular targets to effectively address this issue, especially following the identification of the *D. fragilis* transcriptome, which revealed numerous potential targets for molecular studies (Barratt et al., 2015).

5. CONCLUSIONS

The study employs nested PCR techniques for the rapid identification of *Dientamoeba* directly from clinical samples, demonstrating high sensitivity. The findings suggest that this protozoan should be included in the laboratory diagnosis of patients with diarrhea.

6. ACKNOWLEDGMENTS

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